

The Effect of Various Sunscreen Agents on Skin Damage and the Induction of Tumor Susceptibility in Mice Subjected to Ultraviolet Irradiation

MICHAEL F. GURISH, B.S., LEE K. ROBERTS, PH.D., GERALD G. KRUEGER, M.D., AND
RAYMOND A. DAYNES, PH.D.

Departments of Pathology and Medicine, Division of Dermatology, University of Utah Medical Center, Salt Lake City, Utah, U.S.A.

Sunscreen preparations containing various chemical UV absorbers, para-aminobenzoic acid (PABA), 2 PABA derivatives, benzophenone or a combination of these were topically applied to the backs of C3H/HeN mice prior to their being irradiated with ultraviolet light in the UVB range. In all cases this treatment was effective in preventing the pathological skin changes associated with UVB irradiation. Histological evaluation of skin biopsies from mice treated with the sunscreen preparations and UVB irradiation showed little or no difference from normals in amount of hyperplasia, melanization or parakeratosis present. These histologic changes were observed in animals receiving UVB irradiation in the absence of any sunscreen agent.

Pretreatment with the various sunscreen agents did not, however, prevent the induction of tumor susceptibility as measured by the sustained growth of a UV-induced tumor which is immunologically rejected in normal syngeneic mice. These data show a clear distinction between the effects of UVB irradiation leading to histological changes in the epidermis and those leading to the state of tumor susceptibility in mice. The distinction was further corroborated by the finding that epidermal hyperplasia induced by repeated applications of croton oil had no significant enhancing or inducing effects on the induction of tumor susceptibility. In addition, the induction of tumor susceptibility is not due to wavelengths of light less than 320 nm since this effect was abrogated when the UVB radiation was filtered through glass.

Possible mechanistic differences between the tumor susceptibility generated in UVB and photoprotected UVB irradiated animals were observed, however, when we attempted to adoptively transfer the state of tumor susceptibility to normal animals. While it was readily transferable with splenic lymphoid cells from UVB irradiated animals, all attempts to transfer the tumor susceptibility from photoprotected animals have, to date, been unsuccessful.

It is well known that ultraviolet light (200-400 nm) is capable of producing numerous effects on biologic systems. Environmentally, most of the wavelengths capable of causing direct genetic damage (wavelengths less than 280 nm) are screened out by the atmosphere. However, wavelengths greater than 280 nm are still capable of exerting many potent biological effects.

It is the region between 280 and 320 nm (UVB) that has been found to be primarily responsible for Vitamin D production, melanization, sunburn (erythema and hyperplasia), premature aging of the skin and skin tumors [1,2]. In addition, more recent studies on mice have demonstrated that UVB irradiation is also capable of inducing a subtle alteration in the potential immunological reactivity of the host [3-7]. This change is evidenced by the progressive growth of UV-induced tumor implants in syngeneic mice which have received a subcarcinogenic dose of UV, while a similar implant is immunologically rejected in normal (unirradiated) litter mates. The dose of UV light necessary to induce this tumor susceptible state is at least 10-fold less than the amount of light energy needed to induce an overt tumor. Additional studies have also established that this state is not due to panimmunosuppression. Rather, the underlying mechanism appears to involve the induction and maintenance of regulatory, suppressor T-cells, (T_s) which demonstrate a functional specificity for common tumor associated antigen(s) (TAA) found on all UV-induced tumors [7-8]. These T_s cells are able to inhibit the animal's ability to mount an effective immunological response against the tumor implant resulting in this state of tumor susceptibility [8,9].

Sunscreens, the majority of which employ para-aminobenzoic acid (PABA) (or one of its derivatives) and/or benzophenone as their active ingredient, have been reported to protect against a number of the effects of UV radiation. The ability of topically applied PABA containing sunscreens to reduce the erythema and parallel skin damage caused by prolonged or chronic UV exposure has been amply demonstrated [10-13]. In addition, sunscreens have been shown to protect against both the co-carcinogenic as well as the carcinogenic effects of UV light [12,13]. To date, however, no studies have evaluated the effect of these sunscreen agents on the more subtle and less well understood immunoregulatory modifications now known to be produced by UVB irradiation [3-9].

In this study we report the results of experiments designed to evaluate the potential protective effect of commercial sunscreen preparations on the induction of a tumor susceptible state by UVB. Our results confirm that each of the test agents employed provide excellent protection against the pathologic skin changes associated with chronic UVB exposure. In no case, however, did we observe that a sunscreen preparation was capable of preventing the induction of a tumor susceptible state in mice exposed to UVB irradiation.

MATERIALS AND METHODS

Animals

Four- to 6-week old female C3Hf/HeN mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). All mice were housed at a maximum density of 5 animals per standard 7 × 11 inch cage and maintained on Wayne Sterilizable Lab Blox and acidified water ad libidum. The mice were age matched (6 to 8 weeks old) at the onset of each experiment. All experimental groups contained 5 to 8 animals.

Ultraviolet Irradiation of Mice

The UV light source, energy output and preparation of mice for UV-treatment have been previously reported [4]. Briefly, the UV light

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Reprint requests to: Dr. R.A. Daynes, Department of Pathology, University of Utah Medical Center, Salt Lake City, Utah 84132.

Abbreviations:

PABA: para-aminobenzoic acid

SALT: skin associated lymphoid tissue

SPF: sun protection factors

TAA: tumor-associated antigen(s)

source consists of a bank of 6 FS40 Westinghouse fluorescent sunlamps emitting principally (>60%) wavelengths between 280 and 320 nm with a total energy output of 1.79×10^3 ergs/cm²/sec (0.179 mw/cm²) measured at the dorsal surface level. Measurements were made using an International Light Inc. (Newburyport, Mass. 01950) UV-visible photometer, model #IL200 with detector model #2T101D. Precise measurements of the energy emitted and the calibration of this instrument were performed as previously described [14]. All UV irradiations consisted of 30 min of exposure, 5 times per week. All groups of animals employed in a particular experiment were irradiated concomitantly.

Glass filtered ultraviolet irradiation of animals was done by supporting a square pane of glass (3/16 in. thickness) on blocks of styrofoam just below the fluorescent sunlamps. The cages of mice were placed under the glass so that all light reaching the mice was filtered through the glass. The absorption spectrum of the glass was evaluated by placing a 1 × 4 cm section in a Beckman spectrophotometer model 35 and measuring transmittance at various wavelengths (280–400 nm). Greater than 95% of the light energy below 320 nm was effectively absorbed by employing the glass as a filter.

Treatments

All sunscreen preparations were obtained from commercial sources. The benzophenone-3 (oxybenzone), octyl dimethyl PABA, glyceryl PABA, and the sunscreen base were supplied by Allergan Laboratories (Irvine, California). PreSun (Westwood Pharmaceutical, N.Y.) was used as the source of PABA. Approximate sun protection factors (SPF) for these products were obtained from the supplier or from Sayre et al [15]. The SPF's for the various preparations are: PABA, 12; PABA esters, 13; oxybenzone, 5; PABA esters plus oxybenzone, 15. All the preparations containing PABA or its esters have a SPF of greater than 10, which implies that they absorb greater than 90% of the erythemogenic radiation. The oxybenzone preparation absorbs approximately 80% of the erythemogenic radiation. The various sunscreen preparations were liberally applied (approximately 0.3–0.5 ml) and rubbed on the shaved dorsal surface, ears and tail, one-half hour prior to the UV-irradiation of the test animals.

Croton oil (Sigma Chemical Co., St. Louis, Mo.) was used to induce a state of epidermal hyperplasia. It was first diluted to a 2% solution in acetone, and 50 microliters were applied to the shaved dorsal surface of animals, 3 times per week for 6 weeks.

UV-Induced Tumors and Tumor Challenge

All tumor challenges were performed with RD-87, a C3Hf/HeN, UVB-induced tumor which grows readily in syngeneic UVB irradiated animals but not in normal syngeneic mice [4]. This spindle cell tumor (fibrosarcoma) was maintained *in vivo* by serial subcutaneous passage of 1mm³ tumor fragments into UVB irradiated animals as described previously [4]. All tumor challenges were done in this same manner. Tumor growth rates were determined by measuring 2 perpendicular diameters with calipers twice a week and plotting mean tumor area (the product of the 2 diameters) versus time postimplantation.

Adoptive Transfer of Tumor Susceptibility

Adoptive transfer of the tumor susceptibility with splenic lymphoid cells was performed as previously described [7]. Briefly, spleens were removed, dissociated and washed in media containing 5–10% calf serum. The lymphoid cells were resuspended at 4×10^8 per ml in serum free medium and 0.3 ml injected intravenously via the recipient's lateral tail vein. All adoptive recipients were challenged with a tumor implant within 6 hours following lymphoid cell transfer.

Histology

Animals were randomly chosen from each of the groups and small sections of skin were surgically excised from the ear, and shaved dorsal surface. The samples were surgically excised from the ear, and shaved dorsal surface. The samples were immediately fixed in 10% neutralized formalin. Paraffin sections were stained with either hematoxylin and eosin, or Schmorl's melanin stain and evaluated microscopically for relative amounts of parakeratosis, melanin, hyperplasia and nuclear changes. The nuclear changes observed were peripheral condensation of nuclear material, atypical nuclei, frothy and prominent nucleoli.

RESULTS

The results of the histological evaluation for the amount of hyperplasia, parakeratosis, melanization and nuclear changes seen after three and four weeks of treatment are presented in

Table I. All of the sunscreen agents tested effectively prevented the pathological skin damage associated with UVB exposure. The animals which were photoprotected by prior application of a sunscreen showed no evidence of any parakeratosis or increased melanization, and only slight nuclear changes or increases in the number of epidermal cell layers. The unprotected animals, however, demonstrated marked changes in all of these areas.

An evaluation of tumor susceptibility was made by challenging all test animals with the UV-induced tumor RD87. The data presented in Fig 1 clearly demonstrate that photoprotection of the animals with PABA had no effect on the induction of the tumor susceptible state after 3 weeks of treatment. All test animals were found to be tumor susceptible. Furthermore, the tumor growth rate in the photoprotected mice was equivalent to that seen in the unprotected UVB irradiated animals.

An almost complete lack of protection against the acquisition of tumor susceptibility after 4 weeks of UVB irradiation was also demonstrated in animals treated with the mixture of PABA esters, benzophenone or all 3 in combination (Fig 2). A slight decrease in the tumor growth rate as well as a slight reduction in the percentage of tumor susceptible animals was seen in the animals treated with either the PABA esters alone or in combination with benzophenone. Animals pretreated with PABA, benzophenone or the sunscreen base alone all grew the tumor implant at a slightly increased rate. However, these differences were not significant when evaluated by the Student's *t*-test.

These data suggested that the pathological skin damage associated with UVB exposure does not play a role (or even serve as an indicator) for the induction of tumor susceptibility by UV light. To test for any potentiating or inductive effects due to epidermal hyperplasia, croton oil (2% v/v in acetone) was topically applied to the shaved dorsal surface of mice 3

TABLE I. Evaluation of histologic changes in skin exposed to ultraviolet light with and without the prior application of various sunscreen agents

	No. of cell layers		Para-keratosis ^b	Melanin ^{b,c}	Nuclear changes ^{b,d}
	Epider-mis	Granular layer			
Exp. 1					
Ear ^a					
PABA alone	3.0	1.0	0	1	0
UVB alone	6.0	3.0	4	4	4
UVB & PABA	3.5	1.5	0	1	1
Back ^a					
PABA alone	3.0	1.0	0	0	0
UVB alone	6.0	3.0	3	3	3
UVB & PABA	4.0	1.0	0	0	1
Exp. 2					
Back ^a					
UVB + Benzo-phenone					
+ PABA Esters	3.5	1.5	0	0	0
UVB + PABA Esters	3.5	1.5	0	0	1
UVB + Benzo-phene	3.0	1.0	0	0	0
	23				
UVB + PABA	3.5	1.5	0	0	1
UVB + Sun-screen	6.0	3	2	3	3
Base					
Benzophenone + PABA					
Esters alone	3.0	1	0	0	0

^a Results represent typical histologic findings on the skin of one animal taken at the time of tumor implantation.

^b Quantitated via an arbitrary 0–4 scale: 0 = the amount seen in back skin of animals treated with sunscreen alone; 4 = the amount seen in ear skin of animals treated with UVB alone.

^c Quantitated with Schmorl's melanin stain.

^d Nuclear changes include: Peripheral condensation of nuclear material, atypical nuclei, frothy nucleoli, prominent nucleoli.

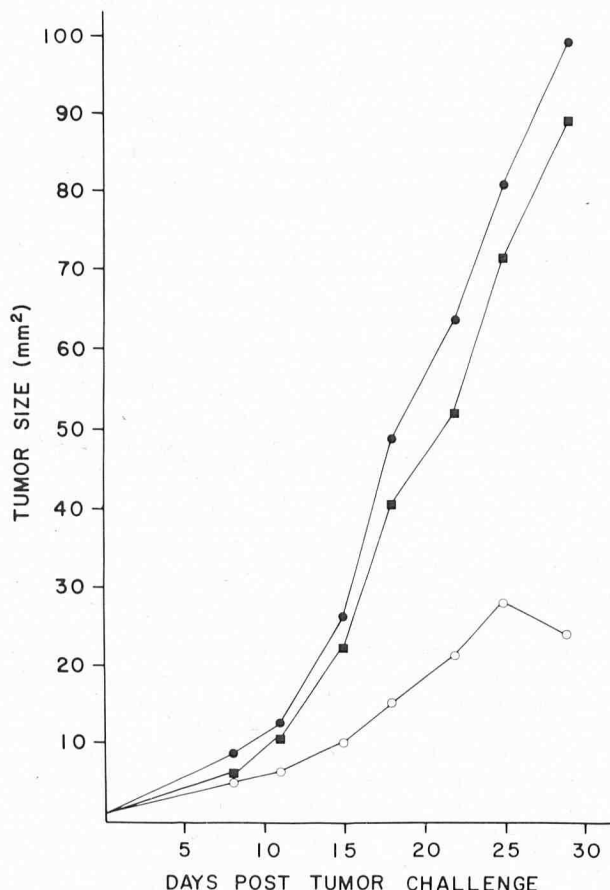


FIG 1. Comparison of growth rates of tumor RD87 in 3 week UVB irradiated animals, one-half of which were protected by prior application of Pre Sun containing 5% PABA. (●) 3-week UVB irradiated mice; (■) Pre Sun applied topically one-half hr before UVB irradiation; (○) Pre Sun applied topically without UVB irradiation. Tumor size is the product of 2 perpendicular diameters. Each point represents the mean of the 6-8 animals in each group.

times per week for 6 weeks. This treatment schedule was performed on 2 groups of mice: (1) normal and (2) age-matched animals given 2 weeks of UVB exposure just prior to beginning the croton oil regimen. For comparison, the 3 other groups in this experiment were: (1) animals given 8 weeks of UVB irradiation to achieve maximal tumor susceptibility, (2) animals given 2 weeks of UVB and then rested for 6 weeks; and (3) normal untreated animals. All groups were implanted with RD87 at the end of the eight-week treatment and subsequent tumor growth rates evaluated (Fig 3). Normal animals with or without the croton oil treatment did not permit tumor growth. Those animals given croton oil after two weeks of UVB did not exhibit a significantly different tumor growth rate from those animals given 2 weeks of UVB and then rested for 6 weeks before tumor challenge. The tumor susceptibility however, was maximal in those animals exposed to 8 weeks of UV as evidenced by the enhanced tumor growth rates in these animals.

The possibility that the induction of tumor susceptibility could be due to wavelengths greater than 320 nm was examined by irradiating the animals with light filtered through glass. The glass was determined to absorb >95% of the light energy below 320 nm. The results of this experiment (Table II) show that animals given 5 weeks of filtered light exposure, even with twice the normal daily dose (60 min) were not tumor susceptible. The control animals (unfiltered exposure) were found to be highly tumor susceptible. This experiment has been repeated with similar findings.

The induction of tumor susceptibility in UVB animals has

also been shown to involve the concomitant induction of suppressor T lymphocytes which are transferable with splenic lymphoid cells [7]. Since PABA plus UVB treated animals were consistently found to be tumor susceptible to the same extent as UVB exposed controls, we examined whether the tumor susceptible state could also be adoptively transferred with spleen cells (Fig 4). Both normal animals given 10^8 splenic lymphoid cells from UVB treated animals and UVB irradiated animals were determined to be tumor susceptible. Those animals given the same number of splenic lymphoid cells from PABA plus UVB treated animals, however, rejected the tumor implant at the same rate as the normal control group. This experiment has also been repeated with equivalent results.

DISCUSSION

UV carcinogenesis is a complex phenomenon, part of which appears to involve a modification in the immunologic potential of the host. Early events which have been elucidated include decreased Langerhans cell function in the epidermis [16], a decrease in antigen presenting cell function in the spleen [17], and the induction and maintenance of a population of Ia^+ suppressor T cells (T_s) [8,9,18]. These T_s cells appear to possess a functional specificity for common, tumor associated antigen(s) found on all UV-induced tumors [8,9]. In addition, UV irradiation, (and by implication the accompanying T_s cells,) has also been shown to be capable of reducing the latency period for tumor induction by a chemical carcinogen administered at an unirradiated site [19]. This observation is distinct from the known cocarcinogenic potential of UV light where the chemical carcinogen is applied at a site receiving UV exposure [12,20], and may explain the observation in man that as many as one-third of all basal cell carcinomas arise on areas receiving very little UV radiation [21]. Furthermore, PUVA (8-methoxypsoralen potentiated UVA) has also been shown to induce tumor

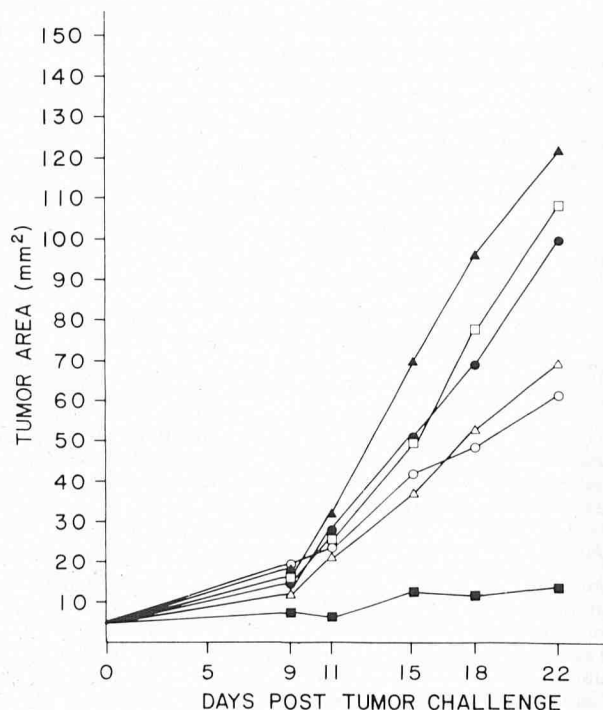


FIG 2. Comparison of tumor RD87 growth rates in animals treated with various sunscreen agents and UVB irradiation. (□) sunscreen base plus UVB; (▲) PABA plus UVB; (●) benzophenone-3 plus UVB; (○) octyl, dimethyl and glyceryl PABA (PABA esters) plus UVB; (△) PABA esters, and benzophenone-3 plus UVB; (■) PABA esters and benzophenone-3 alone. Tumor area is product of 2 perpendicular diameters. Each point represents the mean of the 5 animals in each group.

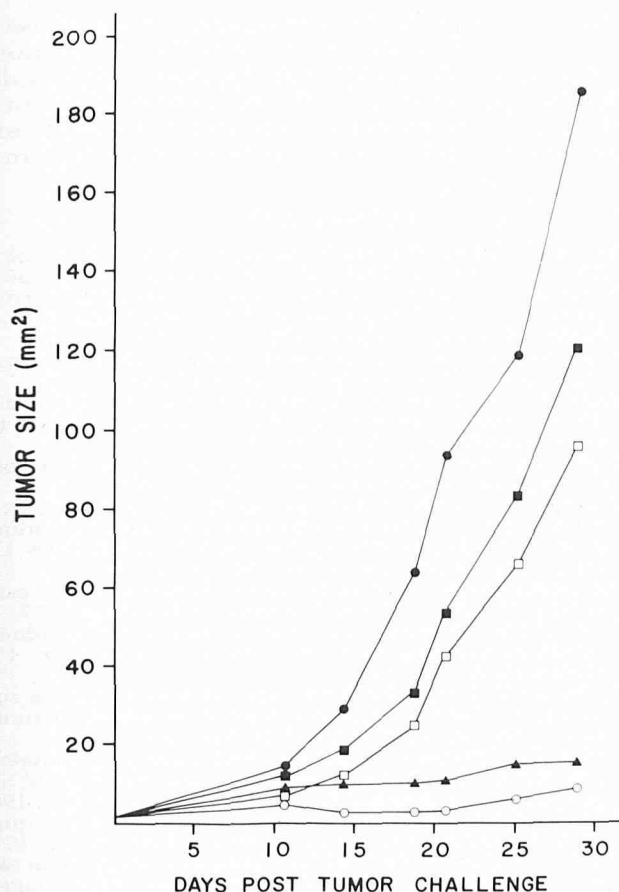


FIG 3. Comparison of tumor growth rates showing a lack of enhancement or induction of the tumor susceptible state due to hyperplasia produced by repeated applications of 2% croton oil. (●) 8-week UVB irradiated animals; (■) 2 weeks of UVB irradiation followed by 6 weeks of 2% croton oil applied 3 times/week; (□) 2 weeks of UVB irradiation, then rested for 6 weeks before tumor challenge; (▲) 2% croton oil applied 3 times/week for 6 weeks; (○) untreated control. Tumor size the product of 2 perpendicular diameters. Each point represents the mean of 6-8 animals.

TABLE II. Abrogation of UVB-induced tumor susceptibility by employing a glass filter to eliminate wavelengths below 320 nm

Group	Treatment ^a	Glass Filtered ^b	# of TBA ^c per # animals challenged
1	30' UVB	-	4/5
2	30' UVB	+	1/5
3	60' UVB	+	0/5
4	-	-	0/5

^a All animals irradiated concomitantly, 5 × per week for 5 weeks.

^b Glass (3/16 in. thickness) was placed over the cages so that all radiation reaching these animals had to first penetrate the glass.

^c Number of animals with progressively growing tumors per number of animals receiving tumor implants. Data collected 25 days after tumor challenge.

susceptibility in mice, an effect which also appears to be mediated by an Ia⁺ cell type [22]. Once again, it has been observed epidemiologically, that patients treated with PUVA show a greater risk of developing a malignancy than an environmentally matched population [23]. Thus, there is an increasing amount of suggestive evidence that UV-induced immunological modifications lead to an increased risk of malignancy. This has led us to investigate the underlying events resulting in, and the effect of photoprotective agents on the induction of the tumor susceptible state in mice.

In addition to its immuno-modulatory influences, UVB also produces numerous skin changes which can be assessed histo-

logically. These include increased melanization, epidermal hyperplasia, parakeratosis and nuclear changes. Previous studies have shown that sunscreen agents are capable of providing good protection against these pathological changes [10-13]. The results presented here confirm and extend these observations, as good protection against these histological changes were noted when any of the sunscreens tested were applied one-half hour before UVB-irradiation. Surprisingly, animals which were photoprotected histologically, were still tumor susceptible as indicated by the progressive growth of the UV-regressor tumor implanted in these animals.

Prior studies have established that the minimum amount of UV treatment necessary to consistently induce the tumor susceptible state is approximately three weeks (30 min per day) and that the average tumor growth rate is proportional to the UVB dose up to approximately eight weeks of irradiation [4]. Our data demonstrate that the observed state of susceptibility in photoprotected animals is equivalent to that observed in the unprotected, irradiated animals, as the tumor growth rates are indistinguishable. This observation, that tumor growth rates are equivalent even though the minimal dose of UVB necessary to establish tumor susceptibility is employed, suggests that sunscreen agents do not significantly affect the mechanism underlying the induction of tumor susceptibility. Thus, the histologic changes in the skin associated with UVB exposure do not appear to play a significant role in the induction of the UVB-induced tumor susceptible state. In further support of this concept is the observation that a chronic state of hyperplasia induced by repeated croton oil treatment does not induce or enhance the tumor susceptible state.

From an initial consideration of the absorption spectrum of PABA, it was suggested that the induction of tumor susceptibility could be due to the wavelengths not effectively absorbed

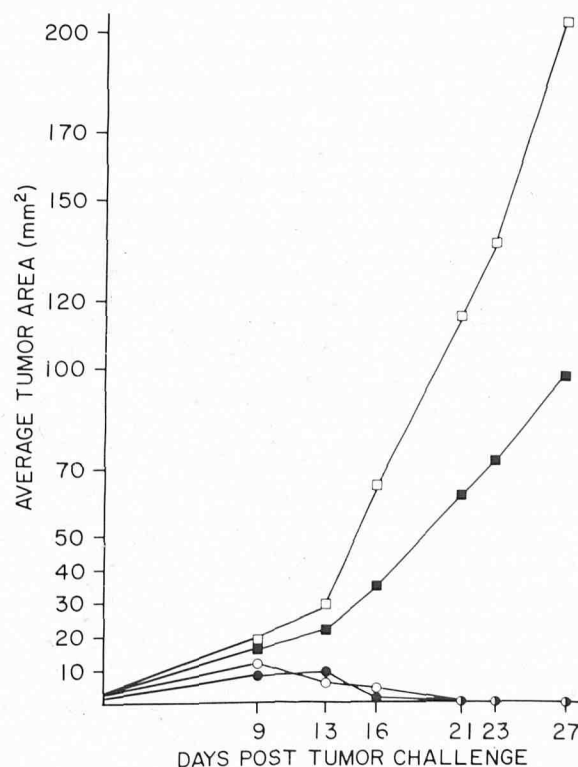


FIG 4. Growth rate of RD87 in animals given splenic lymphoid cells from either UVB or PABA + UVB irradiated animals. (□) 6 week UVB irradiated mice, (■) normals given 10⁸ lymphoid cells from 6 week UVB irradiated mice, (○) normals given 10⁸ lymphoid cells from 6 week PABA + UVB irradiated mice, (●) normal mice. Tumor area is the product of 2 perpendicular diameters. Each point represents the mean of 5 animals.

by PABA. The experiments utilizing benzophenone (which absorbs wavelengths up to approximately 340 nm), glass filters (which eliminate >95% of the light energy below 320 nm) as well as past studies (which have shown that even up to 10 weeks of treatment with UVA light does not induce a tumor susceptible state [22]) demonstrate that this phenomenon is not due to wavelengths of light greater than 320 nm.

Alternate explanations to account for our observations are currently being investigated. One is that the sunscreen agents may act as photosensitizers initiating a photochemical reaction in the skin which leads to the induction of tumor susceptibility. Evidence for this occurring in a prokaryotic system has been presented by Hodges, Moss and Davies [24] who found increased genetic damage when *E. coli* were irradiated in the presence of PABA. Another possibility is that the amount of light energy not absorbed by the sunscreen agents is sufficient to induce the tumor susceptible state.

While tumor growth rates and therefore the degree of tumor susceptibility appears equivalent in both UVB and PABA-UVB treated animals, some differences between the 2 states do exist as shown by the adoptive transfer experiments. Tumor susceptibility induced by UVB is easily transferred to normal syngeneic animals with as few as 3.5×10^7 nylon wool non-adherent T cells [8]. However, our attempts to adoptively transfer the tumor susceptible state using lymphoid cells from photoprotected UVB exposed donors have been uniformly unsuccessful.

Since with photoprotective agents we have essentially eliminated the UVB-induced skin damage and, concomitantly, the adoptively transferable tumor susceptibility, these 2 effects may be related. One possible explanation of this phenomenon is that the inflammation and actinic damage cause a systemic migration of the T_s cells. Thus, while PABA plus UVB (and possibly PUVA) may cause an equivalent number of T_s cells and therefore an equivalent tumor growth rate, the cells might be restricted to skin associated lymphoid tissue (SALT), which would explain our unsuccessful attempts to transfer the susceptibility with splenic lymphoid cells. Evidence for this type of restricted lymphoid circuit comes from a number of sources as previously discussed by Streilein [25].

Another important immunological component found in the epidermis which is affected by both UVB and PABA + UVB is the Langerhans cell. This ATPase positive, dendritic cell in the epidermis shares many characteristics with the antigen presenting macrophage including: expression of Ia determinants as well as Fc and C3b receptors, ATPase and nonspecific esterase positivity, bone marrow origin, and the ability to present antigen to macrophage depleted immune T cells [26-29]. The decreased function of Langerhans cells after UV irradiation has been shown by Toews, Bergstresser, and Streilein [16]. They found decreased numbers of ATPase positive cells in the murine epidermis after UVB irradiation corresponded to a decrease in the ability to contact sensitize the animal at the same site [16]. It was concluded from these data that the antigen presenting function of the Langerhans cell is depressed by UV irradiation. We have extended these observations and found that the same effect is also observed in animals treated with both PABA plus UVB and PUVA (Lynch et al, *J Immunol* in press). The inactivation of the Langerhans cell's antigen presenting capability may play an important role in the early events which lead to UV-induced tumor susceptibility.

In summary, the *in vivo* effects of UVB irradiation are extremely varied, including histologic skin damage, the induction of tumor susceptibility and even overt carcinogenesis. While pretreatment with various sunscreen agents eliminates most of the observed skin damage and significantly retards tumor development, they do not appear to affect the induction of the tumor susceptible state. Evidence indicates however, that the nature of the tumor susceptible state observed in photoprotected animals is not equivalent to that induced in UVB exposed animals since systemic involvement (splenic suppressor cells)

appears to be lacking. Further investigations are in progress to more completely define the tumor susceptible state in photoprotected animals. We are currently involved in determining whether the state is transient and wanes with time (unlike the tumor susceptibility induced in UVB irradiated animals) and also whether it is mediated via a suppressor cell mechanism.

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